Best Practices in Conducting Immunophenotyping Assays Using Spectral Flow Cytometry

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Introduction

Spectral flow cytometry eliminates the need for manual compensation adjustments and allows for the use of fluorochromes with similar peak emissions, augmenting the complexity and versatility of multi-color panels. It also enables better discrimination between autofluorescence and specific signals, thereby enhancing sensitivity, particularly in samples with high autofluorescence.

Although the advantages are significant, different workflows based on this new technology and the increased complexity in panel optimization require increased expertise and practical experiences in spectral flow cytometry to achieve optimized experimental results.

Here, some tips and tricks are presented and discussed, covering **panel design**, **reference control and sample preparation protocols**, **instrument setting**, **unmixing quality control**, **autofluorescence removal**, and **data quality control**.

Experimental

Example data were generated on the Agilent NovoCyte Opteon Flow Cytometer. Flow cytometry data were analyzed by NovoExpress (Opteon) software.

Results and Discussions

Panel design: SAME rules as using traditional flow cytometry

- Reserve bright fluorochromes for low-expressed antigens and dim fluorochromes for high-expressed antigens
- Avoid using similar fluorochromes conjugated with coexpressed antigens
- Minimize fluorochrome spectra overlap, choose fluorochromes with emission signatures as unique as possible

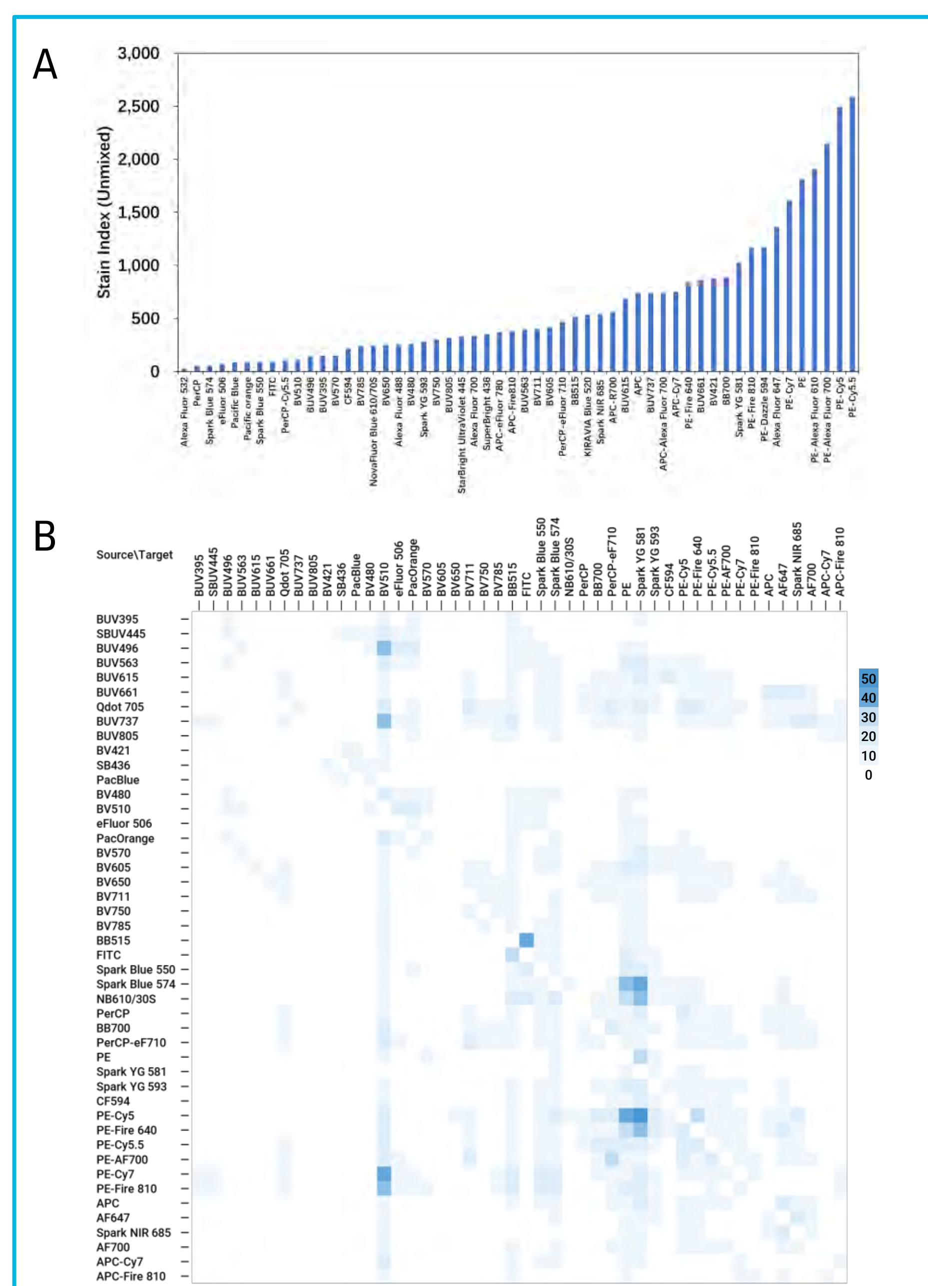

Figure 1. (A) Fluorochrome resolution chart on the Agilent NovoCyte Opteon spectral flow cytometer. Human blood was stained with anti-CD4 antibodies conjugated to the indicated fluorochromes (56 total) and acquired on a NovoCyte Opteon at default settings. The individual single-stains were unmixed by themselves (with autofluorescence).

$$Stain\ Index = \frac{MFI_{Pos} - MFI_{Neg}}{2 \times SD_{Neg}}.$$

(B) Spillover spreading matrix (SSM) for the selected 46 fluorochromes. The samples in (A) were unmixed in the context of the full 46 fluorochromes matrix. The SSM was calculated by NovoExpress (Opteon) software automatically which referred to Cytometry A. 2013 Mar; 83(3):306-15.

Results and Discussions

Reference controls

- Unstained control: required for autofluorescence subtraction; must be the same sample type as the full-stained sample; multiple unstained controls can be easily accommodated in NovoExpress
- Single-stained controls: identical AF signals for negative and positive populations; as bright or brighter than full-stained samples; tandem dye should be from the same lot; using capture beads with evaluation ((Figure 2)

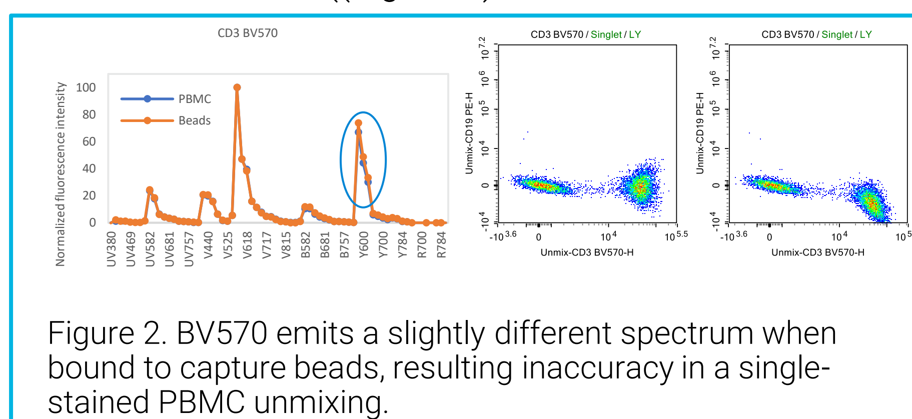

Figure 2. BV570 emits a slightly different spectrum when bound to capture beads, resulting inaccuracy in a single-stained PBMC unmixing.

Sample preparation

- Sample preparation: identical treatments for all sample types (unstained, single-stained, FMO, full-stained).
- Sequential staining can be useful to avoid steric hindrance in large panels (Figure 3).
- Antibody titration on the same model instrument is critical since the newly designed flow cytometers have a high fluorescence intensity and a high sensitivity. For those with strong signals and large spillover spreading errors, appropriately reduce the concentration.
- Fluorochrome aggregates: remove aggregates from antibody stocks by centrifuging 5 min at 16,000 to 18,000 g, 4 °C; Exclude aggregates by gating (Figure 4).

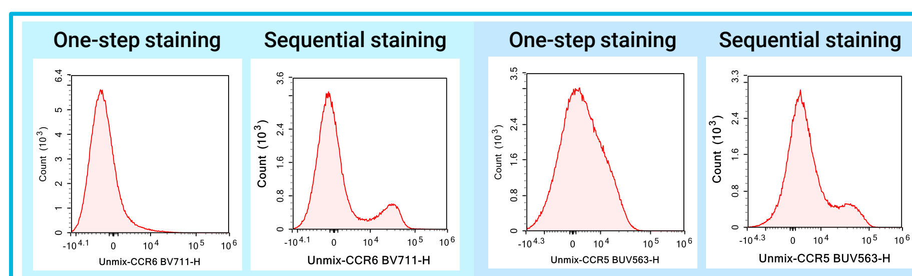

Figure 3. Sequential staining improves the resolution of CCR5 and CCR6 in a 40-color panel.

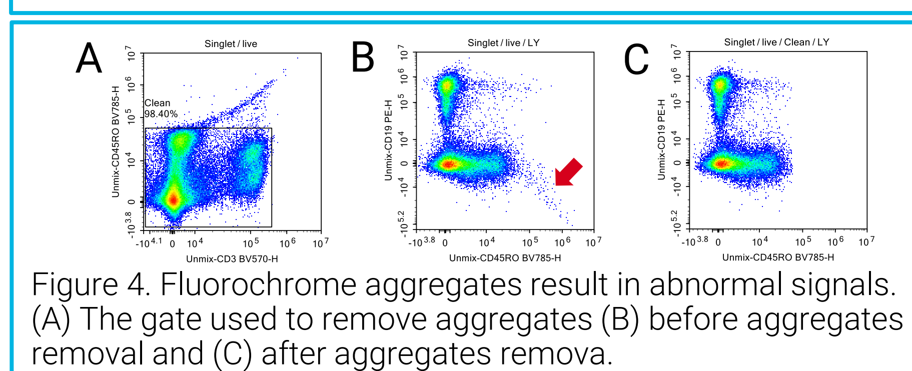

Figure 4. Fluorochrome aggregates result in abnormal signals. (A) The gate used to remove aggregates (B) before aggregates removal and (C) after aggregates remova.

Gain setting

- The Opteon default gains were optimized to balance detector sensitivity and detector dynamic range which are recommended for most assays.
- Signals in all channels must be within scale (ex. not saturated).
- Keep the spectrum signature as expected to minimize the risk of more spillover spreading.

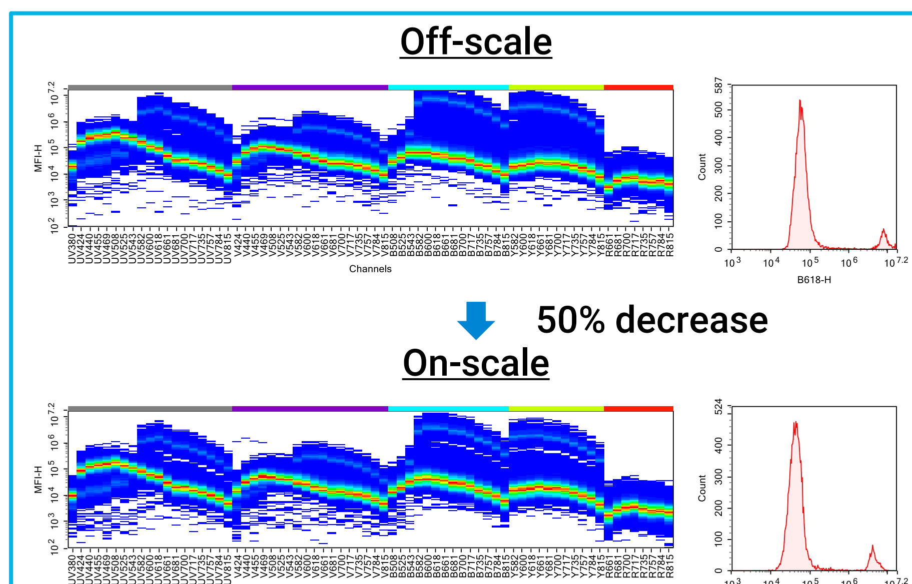

Figure 5. Adjust the gain for all channels with one button click in NovoExpress (Opteon) to decrease the off-scale signals of the PI single-stained sample and keep the spectrum signature.

Results and Discussions

Unmixing quality control

- Check spectral signatures (Figure 6).

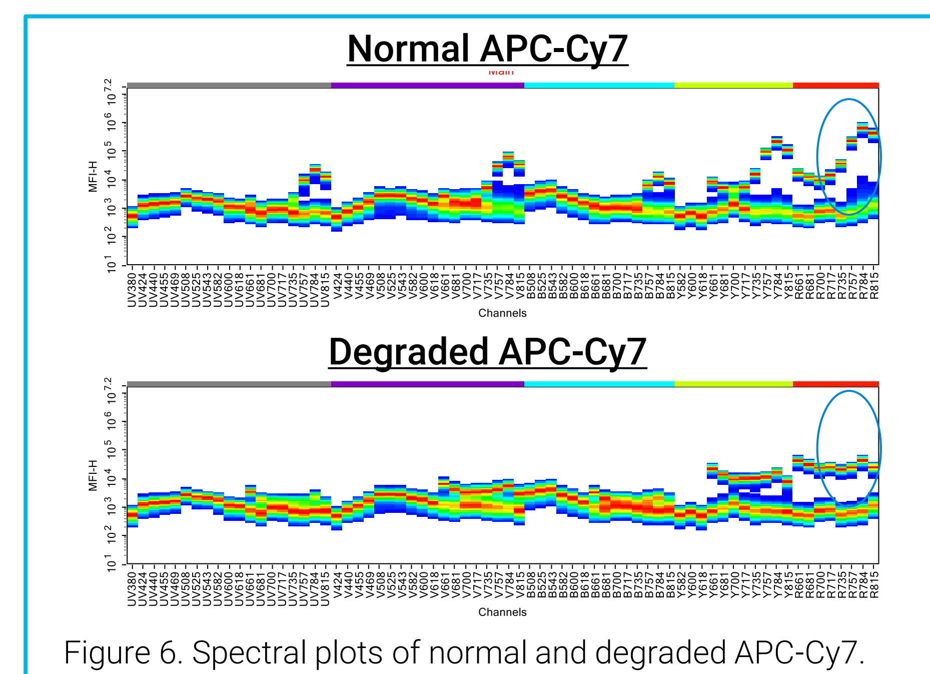

Figure 6. Spectral plots of normal and degraded APC-Cy7.

- Check unmixing of unstained control, single-stained controls, and full-stained samples. There is no positive population for unstained control. The positive and negative populations are well aligned horizontally along the axis for single-stained controls. And there is no super negative population for full-stained samples (Figure 7).

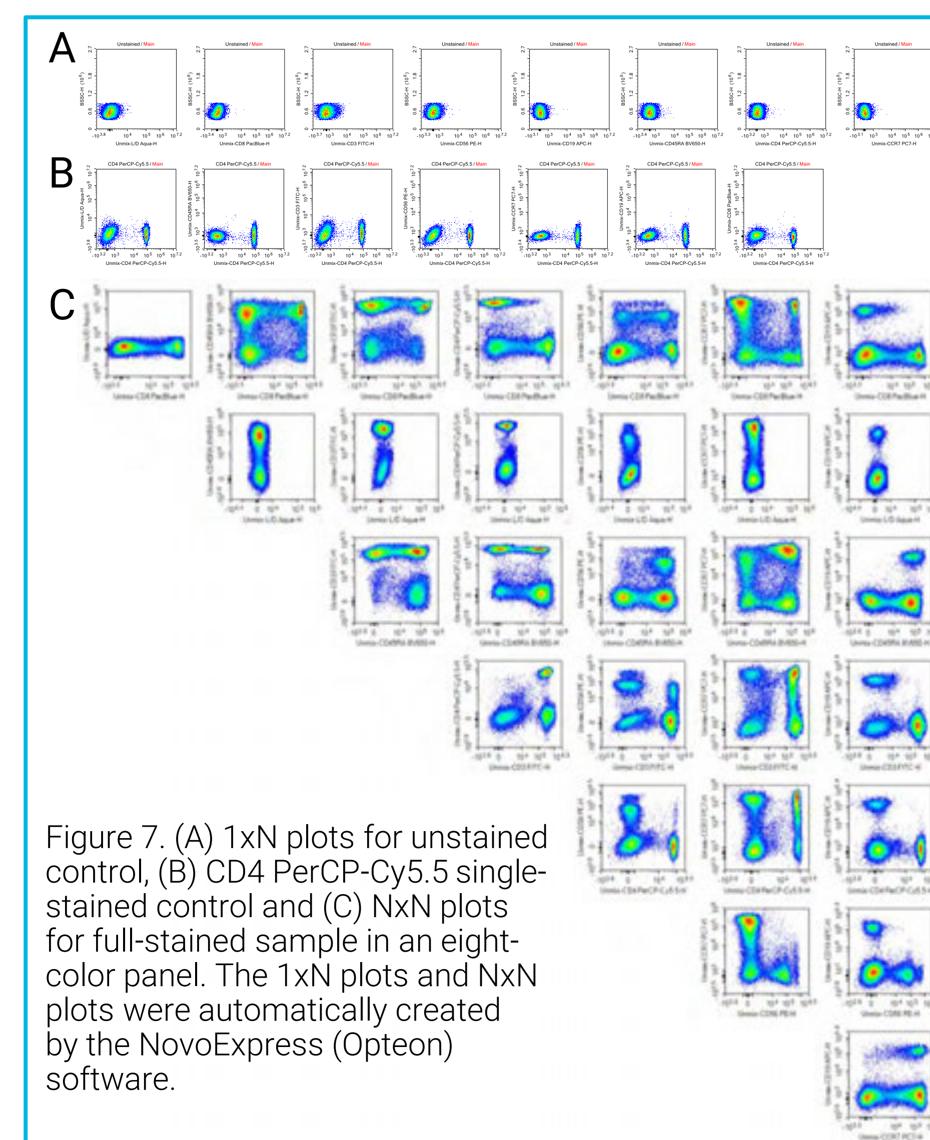

Figure 7. (A) 1xN plots for unstained control, (B) CD4 PerCP-Cy5.5 single-stained control and (C) NxN plots for full-stained sample in an eight-color panel. The 1xN plots and NxN plots were automatically created by the NovoExpress (Opteon) software.

Autofluorescence subtraction

- Identify all heterogeneous AF using an unstained sample prepared identically to a full-stained sample.
- For samples with high AF signal, characterize AF spectrum prior to panel design and consider it as an individual fluorochrome in the panel design.
- Refer to CYTO 2024 poster 46 “Autofluorescence subtraction by spectral flow cytometry” for more details

Conclusions

These tips and tricks can guide users through optimizing a spectral flow cytometry panel design and experimental protocols in scientific research.

References

- Ferrer-Font L, et al. Panel optimization for high-dimensional Immunophenotyping assays using full-spectrum flow cytometry. *Curr Protoc.* 2021;1:e222.
- Richard Nguyen, et al. Quantifying spillover spreading for comparing instrument performance and aiding in multicolor panel design. *Cytometry A.* 2013 Mar;83(3):306-15.

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